Chapter 6

Application of Protein ArrayTubes to Bacteria, Toxin, and Biological Warfare Agent Detection

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Summary

Microarray technology enables the fast and parallel analysis of a multitude of biologically relevant parameters. Not only nucleic acid-based tests, but also peptide, antigen, and antibody assays using different formats of microarrays evolved within the last decade. They offer the possibility to measure interactions in a miniaturised, economic, automated, and qualitative or quantitative way providing insights into the cellular machinery of diverse organisms. Examples of applications in research and diagnostics are, e.g., O-typing of pathogenic *Escherichia coli*, detection of bacterial toxins and other biological warfare agents (BW agents) from a variety of different samples, screening of complex antibody libraries, and epitope mapping. Conventional O- and H-serotyping methods can now be substituted by procedures applying DNA oligonucleotide and antibody-based microarrays. For simultaneous and sensitive detection of BW agents microarray-based tests are available, which include not only relevant viruses and bacteria, but also toxins. This application is not only restricted to the security and military sector but it can also be used in the fields of medical diagnostics or public health to detect, e.g., staphylococcal enterotoxins in food or clinical samples. Furthermore, the same technology could be used to detect antibodies against enterotoxins in human sera using a competitive assay. Protein and peptide microarrays can also be used for characterisation of antibodies. On one hand, peptide microarrays allow detailed epitope mapping. On the other hand, a set of different antibodies recognising the same antigen can be spotted as a microarray and labelled as detection antibodies. This approach makes it possible to test every combination, allowing to find the optimal pair of detection/capture antibody.

Key words: Protein microarray, Antibody microarray, Peptide microarray, Bacterial toxins, Pathogenic bacteria, Biological warfare agents, Antibody screening, Epitope mapping.

1. Introduction

In nature, proteins interact with a variety of different ligands, e.g. DNA, modified and non-modified proteins, and peptides during different fundamental biological processes such as antigen–antibody

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interaction or enzyme–substrate binding. Since the entire set of proteins involved in a biological machinery operates simultaneously, a massive parallel measurement is needed for the analysis and understanding of the system.

Amongst others, two-dimensional gel electrophoresis combined with mass spectrometry, multidimensional liquid chromatography, or protein microarray tests are possible analytical tools with different specificity, sensitivity, and hands-on-time to address such questions. Depending on the used format, protein or peptide microarrays offer the possibility to measure interactions in a miniaturised, economic, automated, and qualitative or quantitative way providing insights into the cellular machinery of a given organism or into the functionality of a protein type *(1, 2)*.

The concept of protein microarrays was inspired by DNA microarrays, which enable the simultaneous detection of thousands of different genes or mRNA molecules for genotyping or expression profiling, respectively *(3)*. It was a logical step to measure the translated and functional products of the genes, using basically the same experimental approach. However, one can not directly transfer all principles from DNA into protein microarray technology. Details such as the type of the reactive surface, spotting procedure, stability of the proteins on the microarray, and the experimental protocol adaptation have to be optimised *(4, 5)*. In the last decade, different protein microarray formats and manufacturing procedures have been developed *(6)* that allow diverse applications in research and diagnostic tests.

O-typing of pathogenic *Escherichia coli*, detection of bacterial toxins and BW agents from a variety of different samples, screening of complex antibody libraries, and epitope mapping are applications that will be discussed in the present contribution.

E. coli is a commensal in the colon of animals and humans, and some of the serotypes or pathotypes are associated with diseases such as meningitis, urinary tract infection, diarrhoea, and even septicaemia. O-serotyping is an established method, which is based on variable highly immunogenic lipopolysaccharides (LPS) on the cellular surface. It has been shown previously that conventional O- and H-serotyping methods can be substituted by DNA oligonucleotide and antibody-based microarrays, which consume less time and resources *(7, 8)*.

A further application arises from the growing threat of terroristic attacks and nations' vulnerability to BW agents including toxins, viruses, and bacteria. Here, protein microarrays might be used to analyse environmental and air samples as well as to constantly monitor food and water supplies. Additionally to a sensitive and definite identification of single agents, protein arrays with carefully selected antibodies allow the parallel detection of BW agents and can distinguish closely related pathogens *(9–12)*.

Compared with DNA arrays, their application is not restricted to the detection of nucleic acid carrying pathogens. They can also be used to detect toxic substances such as ricin or bacterial exotoxins, e.g., staphylococcal enterotoxins in food and environmental matrices *(13)*. Additionally, an antibody array might be used in a competitive assay to detect specific antibodies in human sera, indicating a previous exposure to a given antigen.

The most critical step in microarray assays comprises sample preparation and labelling, which is easier for protein applications than for DNA array-based tests. However, the challenge is to find the optimal combination of capture and detection antibody for a specific antigen. Because of the parallel detection of different agents that might be expected even within a single specimen, cross reactivities of the different labelled detection antibodies have to be ruled out although a high overall sensitivity of the complete test regarding each target needs to be retained. The very concept of an array offers the possibility to screen all available antibodies for a specific antigen by using them as both capture antibody to be spotted and detection antibody to be labelled. By testing every possible combination, the detection/capture antibody pairs best suited in terms of specificity and sensitivity can be easily detected. After performing these experiments, the best combination of antibodies for each distinct target can be used for the routine application of the assay, or for further optimisation.

Another experimental option is to immobilise antigens (peptides as single epitopes or even complex molecules) instead of antibodies. Microarrays with spotted peptides can so be used for detailed epitope mapping *(14)*.

Altogether protein microarrays offer various opportunities in research and diagnostics, which might, combined with the appropriate protocols and platforms, change our understanding of the nature of biological processes, and which could help to perform diagnostic tasks in a faster, more reliable, and economic way.

2. Platforms, Microarrays, and Detection and Assay Principle

2.1. Platforms

For the techniques and protocols discussed later, a microarray platform was combined with a unique detection principle (**Fig. 1**). The ArrayTube system is a 1.5 ml reaction vial where a 3 mm × 3 mm glass microarray with a spotted area of 2.4 mm × 2.4 mm is mounted onto the bottom of a via that is compatible to standard laboratory equipment such as thermomixers (http://www.clondiag.com/products/array_tube/index.php). For automation of such tests, the ArrayStrip system

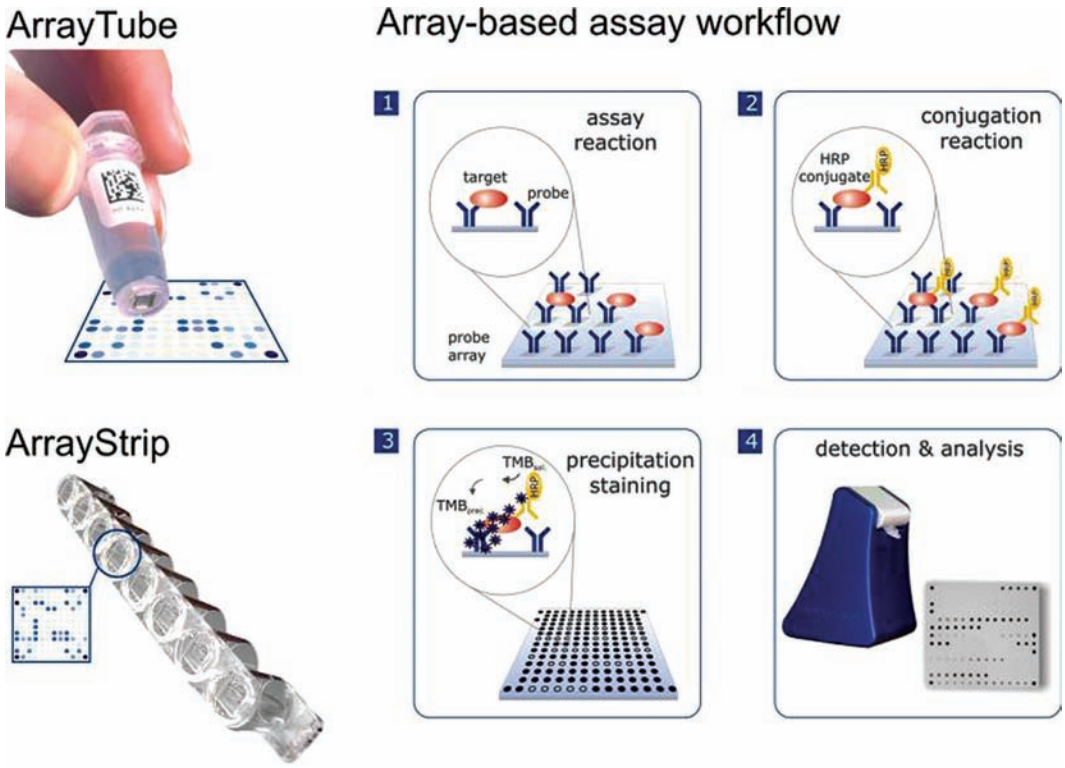


Fig. 1. Within the ArrayTube platform 3 mm × 3 mm pre-spotted microarrays are mounted onto the bottom of 1.5 ml reaction vessels that are used manually with existing lab equipment. The ArrayStrip platform contains 4.2 mm × 4.2 mm microarrays that can be completely processed and analysed in an automated device. In both platforms the workflow and analysis principle is the same. A directly or indirectly (via a secondary antibody) biotinylated antigen binds to a previously spotted and covalently coupled capture antibody. In a subsequent step, HRP catalyses a local TMB substrate precipitation, which can be detected simply by transmission measurement.

(http://www.clondiag.com/products/array_strip/index.php) was developed. It comprises eight microarrays of 4.2 mm × 4.2 mm and a spotting area of 3.4 mm × 3.6 mm, which are included in one strip. Twelve strips can be combined and used as one 96-well microtiter plate.

2.2. Microarrays

Protein/peptide microarrays were produced using an three dimensional epoxy-modified glass as spotting substrate. Substances were spotted without any detergents using final concentrations between 0.05 and 1 mg/ml protein or 1 and 5 mg/ml peptide (Jerini, Berlin, Germany) in 1 × phosphate buffered saline (PBS) containing 5–20 mM sucrose or trehalose. All peptides used consist of one C-terminal glycine residue and three N-terminal lysine residues combined with a trimesyl-tris(3,5-dibromo)salicylate

spacer (Jerini, Berlin, Germany). To avoid the disadvantages of piezo-based and split-pin-based spotting (e.g., undefined spotting volumes, cross contamination of substances due to incomplete washing procedures, "ghost spots" caused by dust particles, and waste of expensive spotting solutions of which always a portion remains in microtiter plates or cartridges that finally will be discarded) a newly developed contact spotting procedure was generally applied. Each substance to be spotted has its own spotting needle capillary, which is suited for both spotting and storage. Because of this approach, the complete amount of protein solution can be spotted without wasting any substance and without the possibility of cross contamination. After manufacturing, protein microarrays were sealed under argon atmosphere into nontransparent bags and stored at 4°C or even room temperature until usage.

2.3. Detection Principle

For both possible platforms shown in **Fig. 1**, the labelling technology is based on a catalytically induced tetramethylbenzidine (TMB) precipitation, which directly correlates to the amount of target molecules binding to the probe array. Analysis of the resulting precipitation patterns is done by simple CCD-based transmission measurements within a dedicated reader.

The resulting pictures were always analysed automatically using the software Iconoclust in combination with the Partisan ArrayLIMS System Database and defined scripts (CLONDIAG, Jena, Germany) for the different microarray layouts used. Spots were detected, and mean values of the spots (MV) as well as the local background (BG) were measured. Normalised signal intensities (NSI) were calculated by the equation NSI = 1 – MV/BG. Using this algorithm, changes or inhomogenities in background intensity can be eliminated resulting in better comparability between different experiments. Assay-specific threshold values for interpretation as positive/negative were defined using reference samples characterised with another method.

2.4. Assay Principles

2.4.1. Serotyping of Escherichia coli

O-serotyping is used routinely as a presumptive guide to distinguish between pathogenic and commensal *E. coli*. Compared with conventional serotyping assays, protein arrays can produce O-serotyping results more efficiently considering the high costs of typing sera ***(8)***. Such sera, which have been raised against *E. coli* pathogenic to both humans and animals and which are used routinely for O-serotyping, were immobilised on the surface of protein microarrays. The two basic types of the assay and three resulting images are shown in **Fig. 2**.

2.4.2. Antibody Screening and Epitope Mapping

To screen a defined set of antibodies against an antigen for optimal specificity and sensitivity, different concentrations of the antibodies (sometimes diluted in an inert protein such as BSA) are

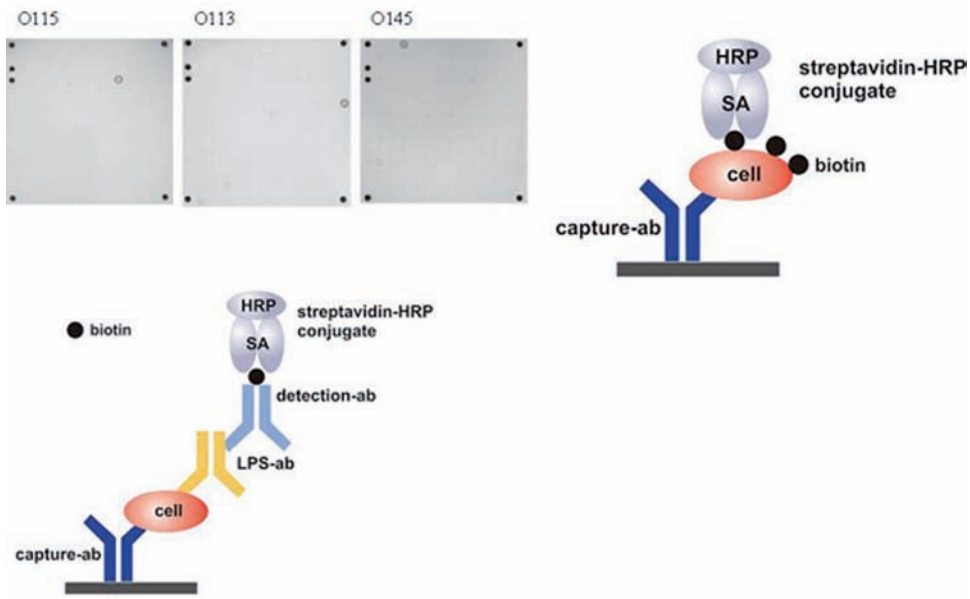


Fig. 2. Two different experimental set ups for *E. coli* O-typing are shown. The indirect labelling procedures using LPS monoclonal antibody WN1222-5 *(15, 16)* and biotinylated anti-mouse IgG (Sigma 8520) are described in *(8)*. In the second labelling procedure, complete *E. coli* cells are biotinylated directly. Three pictures of processed arrays after reaction with *E. coli* O-Types O113, O115, and O145 are shown. The microarray contains 176 different, single spotted LPS-specific sera in a 14 × 14 grid layout.

combined and immobilised on the same array. In addition, all spotted antibodies are labelled individually with, e.g., biotin or HRP. Every antibody can either be used as a covalently coupled capture antibody on the array or as a labelled detection antibody (**Fig. 4**). By testing the resulting microarrays with an appropriate concentration of the detection antibody and different concentrations of the antigen, the optimal antibody pair in terms of specificity and sensitivity can be found. Even an approximate antigen quantification can be performed using this approach (**Fig. 4**).

To characterise a specific monoclonal antibody, peptide arrays can be used for epitope mapping. For this application, a set of overlapping peptide sequences is derived from the original amino acid sequence of the given protein. These peptides are to be synthesised and subsequently immobilised as a microarray. If a labelled antibody interacts with such an array, it will bind preferentially to its epitope peptide (**Fig. 3**).

2.4.3. Detection of Staphylococcal Enterotoxin B

Staphylococcus aureus is a gram-positive bacterium, which can cause skin and soft tissue infections, pneumonia, and septicaemia. It also produces a variety of toxins, which manipulate or disrupt functions of the host immune system. Among them there are

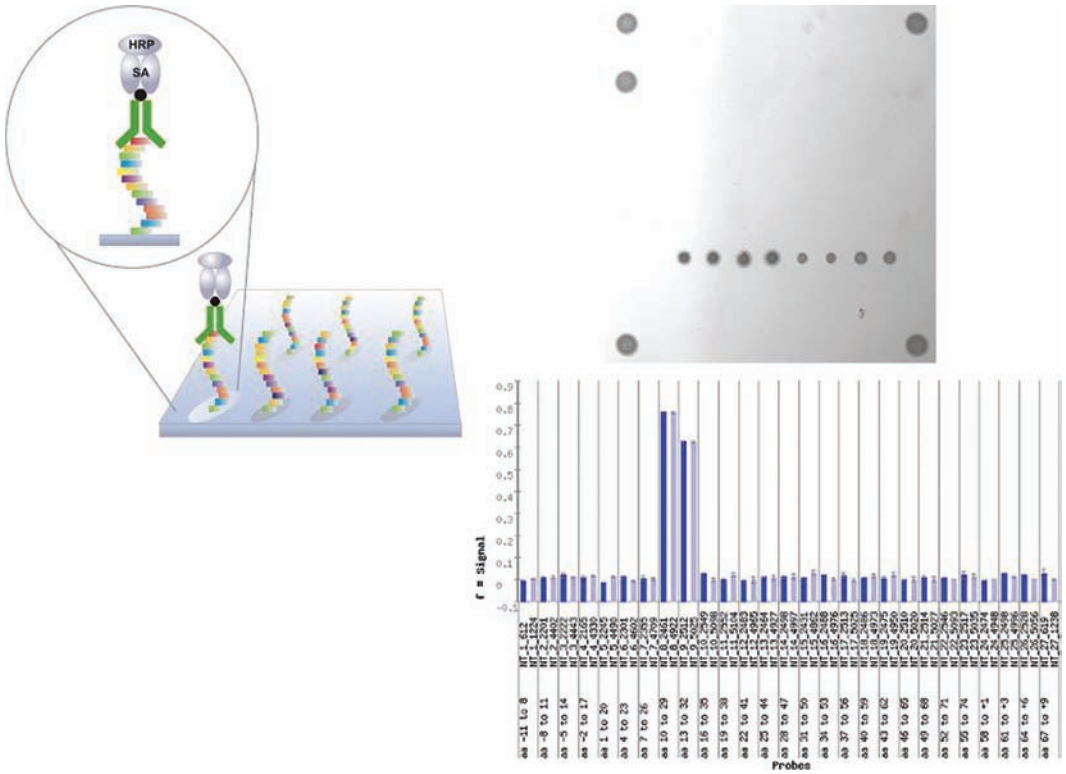


Fig. 3. A biotin-labelled antibody specific for an antigenic protein is applied within an array-based assay, where the used microarray contains multiple and overlapping peptide sequences resulting from a clustering of the antigen. In the shown example, a 76 amino acid protein is mapped, by spotting 27 overlapping peptides on a microarray. All peptides are immobilised in duplicate and in two different concentrations. The results of the interaction between the labelled antibody and all covalently attached peptides show clearly that the epitope is situated between amino acid positions 10 and 32.

so-called enterotoxins, which induce, when ingested, intense but transient vomiting, diarrhoea, and general malaise *(17)*. These heat-stable toxins are a common cause of food poisoning as *S. aureus* is able to grow in foodstuffs after being inoculated by, e.g., infected lesions at the hands of kitchen staff. Because of their incapacitating effect, they also have been investigated as a possible agent in biological warfare *(9, 11, 18)*.

The assay described earlier was designed to detect, beside others, one of the more common enterotoxins, enterotoxin B (*seB* or *entB*). The toxin is bound to spotted, i.e., immobilised, monoclonal antibodies or sera, and detected using a biotinylated monoclonal antibody (which yields the specificity of the assay) and a precipitation reaction as described earlier. The assay can be used either to detect enterotoxin B directly from homogenised foodstuffs or to screen staphylococcal cultures for their ability to produce the toxin (**Fig. 5**). An application for the detection of neutralising antibodies in human sera by competitive

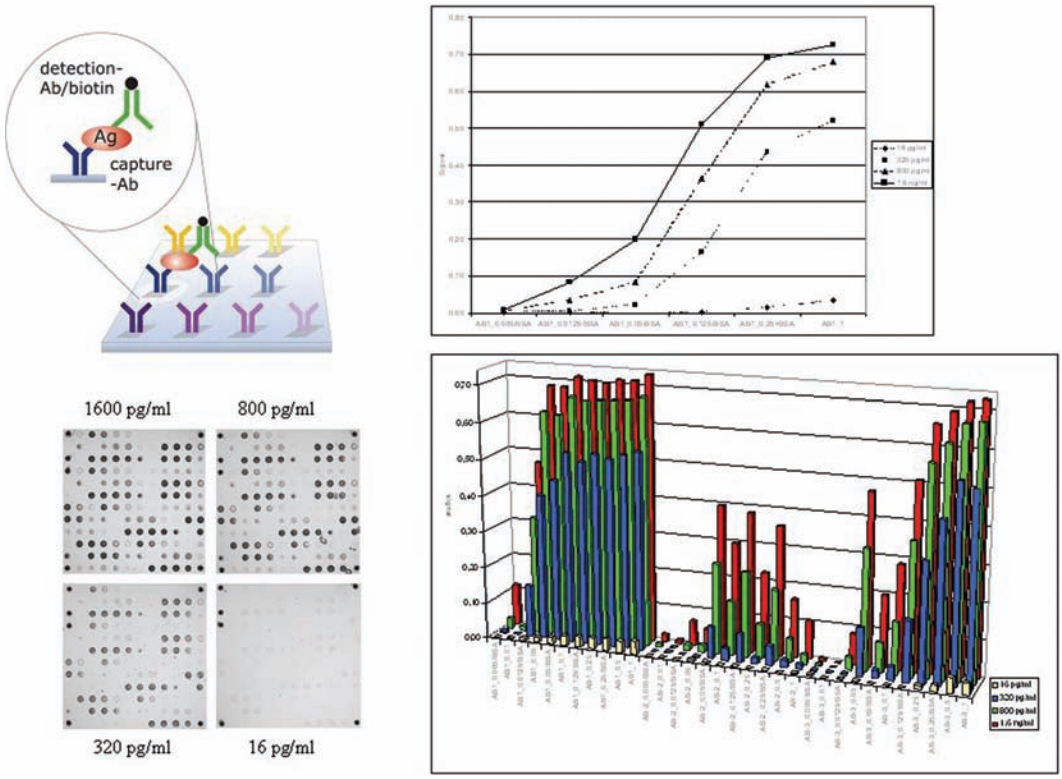
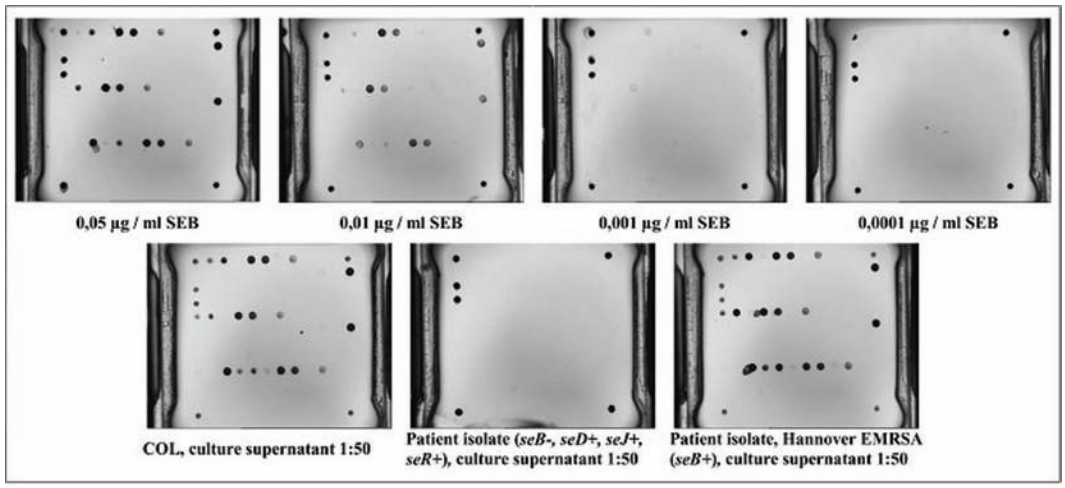


Fig. 4. Three different antibodies specific for C-reactive protein (CRP) are spotted in two different dilution series for each antibody in a 12 × 12 layout, four times redundant and mounted in ArrayTubes. One dilution series contains decreasing antibody concentrations whereas the other one consists of constant protein but decreasing antibody concentrations. For the additional protein component, BSA was used. As shown in the diagram, the resulting microarrays were processed with characterised human serum diluted (between 1:1,000 to $1:10^6$) in 1 × PBS to end concentrations of CRP of 16, 320, 800, and 1,600pg/ml. The detection is performed using a biotinylated secondary antibody (which was the same as one of the three spotted antibodies). The resulting microarray pictures show a detection limit near 16pg/ml. Furthermore, the 3D bar plot of all clustered results of the four images enables the shown diagram with the calibration curve for rough quantification of CRP within one microarray experiment.

binding is investigated. The protocols refer to the ArrayTube system (see later).

2.4.4. Detection of Biological Warfare Agents

For the specific detection of BW agents the ArrayTube™ platform is used and diverse antibodies against such agents including toxins, viruses, and bacteria are screened (**Fig. 6**). Commercially available antibodies as well as monoclonal antibodies prepared from hybridoma cells ***(9, 21–25)*** and polyclonal antibodies raised according to standard procedures ***(26)*** were manufactured and spotted as described earlier. After incorporation of the microarray into the ArrayTube, the tubes were stored under argon at 4°C. For more details concerning the applied antibodies refer to ***(9)***.



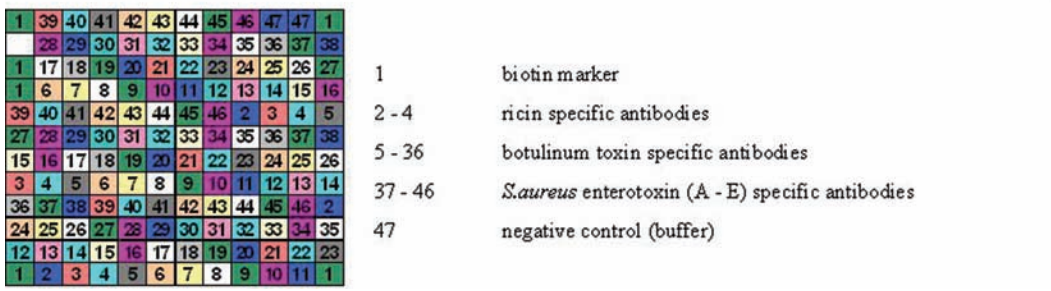


Fig. 5. The applied array contained 47 different substances spotted in a 12 × 12 layout in threefold redundancy. The substances contain a biotin marker as positive control, buffer spots without protein as negative control, and several antibodies in varying concentrations with different specificities for ricin, botulinum toxins, and *S. aureus* enterotoxins A, B, C, D, and E. The figure shows the detailed layout and serial dilution of recombinant *S. aureus* enterotoxin B and several culture supernatants using the array. The specificity of the assay for enterotoxin B results from the selection of the labelled detection antibody (see text).

3. Materials

3.1. O-Serotyping of Escherichia coli

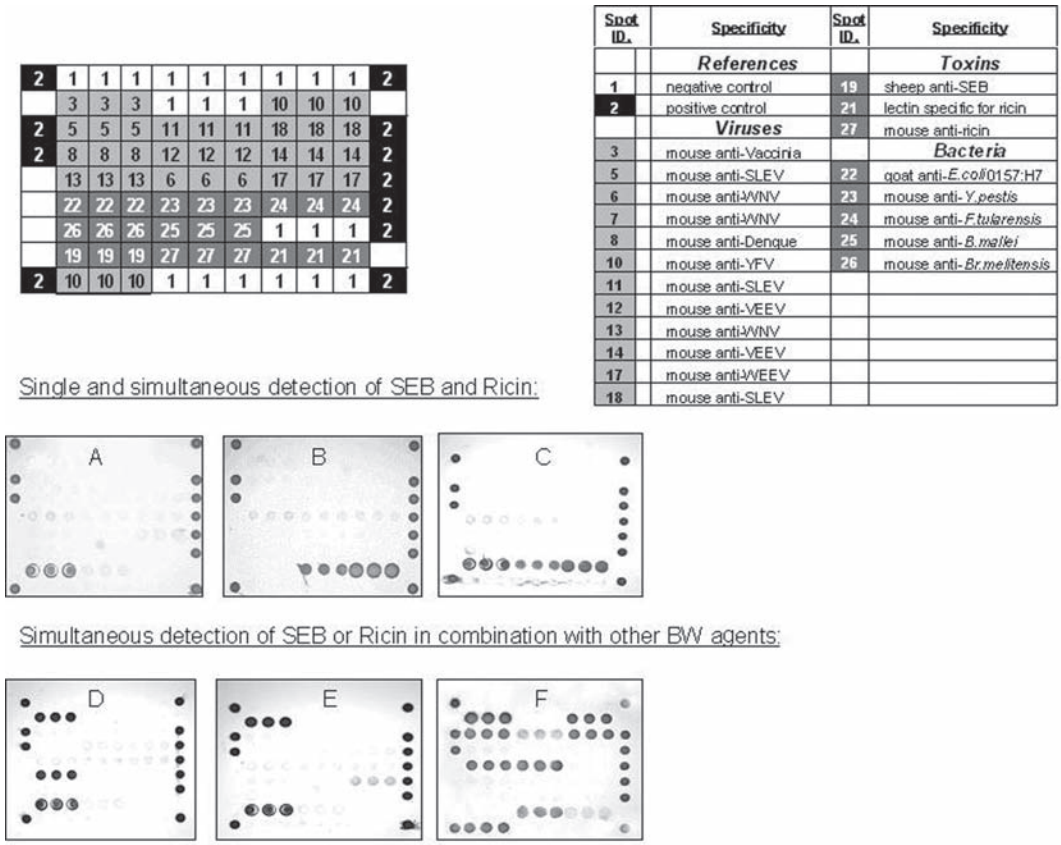
Instruments:

Eppendorf biophotometer: Eppendorf, Hamburg, Germany.

Rotary shaker: Thermomixer comfort, Eppendorf, Hamburg, Germany.

atr01 or atr03 reader: CLONDIAG, Jena, Germany.

YM-30 columns: Millipore, Schwalbach, Germany.



Spot ID.	Specificity	Spot ID.	Specificity
	References		*Toxins*
1	negative control	19	sheep anti-SEB
2	positive control	21	lectin specific for ricin
	Viruses	27	mouse anti-ricin
3	mouse anti-Vaccinia		*Bacteria*
5	mouse anti-SLEV	22	goat anti-*E.coli*0157:H7
6	mouse anti-WNV	23	mouse anti-*Y.pestis*
7	mouse anti-WNV	24	mouse anti-*F.tularensis*
8	mouse anti-Denque	25	mouse anti-*B.melitensis*
10	mouse anti-YFV	26	mouse anti-*Br.melitensis*
11	mouse anti-SLEV		
12	mouse anti-VEEV		
13	mouse anti-WNV		
14	mouse anti-VEEV		
17	mouse anti-WEEV		
18	mouse anti-SLEV		

Fig. 6. Shown are representative ArrayTube results obtained for a protein chip after performing single and parallel analysis of different BW agents. From each analysis the 60th picture is presented. Concentrations of analytes in experiments A–F were as follows: A: SEB (2ng/ml), B: ricin (1ng/ml), C: SEB (5ng/ml) and ricin (2ng/ml); D: Vaccinia virus (10^5TCID$_{50}$/ml), *E. coli* O157:H7 (5×10^4cfu/ml) and SEB (2ng/ml); G: Vaccinia virus (10^5TCID$_{50}$/ml), *E. coli* O157:H7 (5×10^4cfu/ml), *Francisella tularensis* (10^7cfu/ml) and SEB (5ng/ml); E: Vaccinia virus (10^5TCID$_{50}$/ml), *Francisella tularensis* (10^7cfu/ml) and SEB (2ng/ml); I: yellow fever virus 17 D(10^5TCID$_{50}$/ml), West Nile virus NY (10^3TCID$_{50}$/ml), St. Louis encephalitis virus (2×10^7TCID$_{50}$/ml), Vaccinia virus (10^5TCID$_{50}$/ml), and ricin (2ng/ml). The following biotinylated ([b]) detection antibodies were used: A: [b]antiSEB, B: bRCH1, C: [b]RCH1, [b]anti-SEB1; D: [b]5B1, [b]anti-*E. coli*, [b]anti-SEB; E: [b]5B1, [b]antiSEB, [b]FT140/11/1/06; F: [b]4G2, [b]WNV MAB8151, [b]5B1, [b]SLEV MAB 8744, [b]RCH1. For details about the applied antibodies refer to *(10)*.

Solutions:

1. 2 × TY broth: 1 l of 2 × TY broth containing 16 g tryptone/peptone, 10 g bacto yeast extract (both BD, Heidelberg, Germany), 5 g NaCl.
2. 2.2 mg NHS-LC-biotin (Pierce, Boston, MA, USA) is stored at −20°C and is (always freshly) dissolved in 400 µl double-distilled (dd) water.
3. PBS–Tween wash buffer: 1 × PBS with 0.01% Tween20.

4. PBS–Tween–FCS *(10)*: 90 µl 1 × PBS–Tween plus 10 µl foetal calf serum.
5. PBS–FCS–Tween: 1 × PBS with 0.01% Tween20 and 1% FCS.
6. Anti-*E. coli* core LPS monoclonal antibody WN1222-5 *(15, 16)*: diluted to 52ng/ml in PBS–FCS–Tween.
7. Secondary biotinylated anti-mouse IgG: Sigma 8520, diluted 1:10,000 in PBS–FCS–Tween.
8. Streptavidin-poly-horseradish peroxidase (SA-poly-HRP, Pierce, Boston, MA, USA) is diluted in PBS–FCS–Tween to 100pg/µl.
9. Seramun green (Seramun, Woizig, Germany) – precipitation substrate of streptavidin-poly-horseradish peroxidase.

3.2. Antibody Screening and Epitope Mapping

Instruments

1. Photometer: Nanodrop ND1000, PeqLab, Erlangen, Germany.
2. Rotary shaker: Eppendorf, Hamburg, Germany.
3. atr03 reader in combination with the Iconoclust software package and an assay specific script: CLONDIAG, Jena, Germany.
4. Microcon YM-30 column: Millipore, Schwalbach, Germany.
5. ZEBA spin columns: Pierce, Boston, MA, USA.

Solutions

1. 2.2 mg NHS-LC-biotin (Pierce, Boston, MA, USA) is stored at –20°C and is always freshly dissolved in 400 µl dd water.
2. PBS–Triton: 1 × PBS buffer containing 0.5% Triton X100.
3. Streptavidin-buffer: 1 × PBS, 1% BSA, 0.5% Triton X100, and 3 µg/ml poly-HRP-streptavidin (Pierce, Boston, MA, USA).
4. True Blue peroxidase substrate: Medac, Hamburg, Germany.
5. PBS–FCS–Tween: 1 × PBS, 0.05% Tween, 1% FCS.
6. PBS–Tween–FCS *(10)*: 90 µl 1 × PBS plus 10 µl FCS plus 0.05% Tween.
7. Streptavidin poly-HRP (Pierce, Boston, MA, USA) concentration of 0.2ng/ml in 100 µl PBS–FCS–Tween.
8. Seramun green (Seramun, Woizig, Germany) precipitating substrate.

3.3. Detection of Staphylococcal Enterotoxin B

Instruments

Rotary shaker: Thermomixer Comfort, Eppendorf, Hamburg, Germany.
atr01 reader: CLONDIAG, Jena, Germany.

Solutions

1. Medium [Noda et al. ***(19)***]: 25 g yeast extract, 20 g casamino acid, 20 g sodium glycerophosphate, 0.64 g $FeSO_4 \times 7H_2O$, 0.64 g citric acid, 6.25 g $Na_2HPO_4 \times 12\ H_2O$ (or alternatively, 3.65 g $Na_2HPO_4 \times 1\ H_2O$), 400 mg KH_2PO_4, 20 mg $MgSO_4 \times 7\ H_2O$, 10 mg $MnSO_4 \times 4\ H_2O$, 19.8 mg sodium lactate solution (50%), and add to 1 l finale volume with water.
2. PBS: 1.2 g $NaH_2PO_4 \times H_2O$, 4.4 g Na_2HPO_4, and 8.2 g NaCl, as well as 1,000 ml de-ionised, sterile water. Its pH value is adjusted to 7.4 using 1 M NaOH.
3. PBS–Tween: 50 µl Tween20 in 100 ml PBS.
4. PBS–Tween–BSA: 3 g bovine serum albumin in 100 ml PBS–Tween.
5. Antibody BBC202 (Toxin Technology, biotinylated as described earlier; 1:2,000 in PBS–Tween).
6. Streptavidin-HRP: Streptavidin-HRP (Pierce, Boston, MA, USA) needs to be stored at –20°C. A stock solution of 1:100 in PBS buffer can be kept at 4°C for 5–7 days. Prior to use, this stock solution is to be diluted to 1:100 in PBS–Tween–BSA. This working solution can not be stored.
7. Seramun Green (Seramun, Woizig, Germany) – precipitating substrate.

3.4. Detection of Biological Warfare Agents

Instruments

Horizontal tube shaker: Eppendorf, Hamburg, Germany.
atr01 reader: CLONDIAG, Jena, Germany.
PD-10 columns: GE Healthcare, Munich, Germany.

Solutions

1. All viruses used represent models for BW relevant Flavi- or Alphavirus species, and are part of the collection of the German Armed Forces Scientific Institute for Protective Technologies (WIS).
2. Bacterial strains are either part of the collection of the German Armed Forces Scientific Institute for Protective Technologies or are available from the American Type Culture Collection (ATCC).
3. Staphylococcal enterotoxins: Toxin Technology, Inc., USA.
4. Biotin-NHS ester: Vector Laboratories, Burlingame, CA, USA or Sigma-Aldrich Chemie GmbH, Munich, Germany.
5. Streptavidin-HRP conjugates: GE Healthcare, Munich, Germany.
6. PBS–Tween: 1 × PBS + 0.01% Tween20.
7. PBS–FCS–Tween: 1 × PBS + 0.1% FCS + 0.01% Tween20.

8. Streptavidin-poly-horseradish peroxidase (SA-Poly-HRP, Pierce, Boston, MA, USA): 1:10,000 dilution.
9. TMB: CLONDIAG, Jena, Germany.

4. Methods

4.1. O-Serotyping of Escherichia coli

4.1.1. Bacterial Culture, Antigen Preparation, and Labelling

1. Starting from a single colony on solid growth media, *E. coli* cells are grown in 2 × TY broth overnight to stationary phase at 300 rpm and 37°C.
2. OD (600 nm) of an adequate dilution (culture diluted in 2 × TY broth) is measured and calculated using the Eppendorf biophotometer according to manufacturers' instructions to compare the growth of different strains.
3. An aliquot of 2.6 ml of the overnight culture is mixed with 60 µl 37% HCHO and subsequently incubated on a rotary shaker at 65°C with 300 rpm for 10 min.
4. The cells are centrifuged at room temperature and 6,000 rpm for 10 min, and the supernatant is discarded. The cell pellet is dried and re-dissolved in 1.3 ml 1 × PBS buffer and stored at 4°C until use in the O-typing assay or direct labelling with NHS-biotin.
5. Twenty microlitres of the *E. coli* cells are mixed with 30 µl 1 × PBS and 5 µl of the freshly prepared NHS-LC-biotin solution, vortexed, sonicated for 5 min, and incubated at 21°C and 550 rpm for 45 min.
6. The biotinylated cell mixture is purified using YM-30 columns by the addition of 350 µl 1 × PBS to 50 µl sample, placed on the column, and centrifuged at 14,000 rpm for 9 min. The column is subsequently washed twice with 400 µl 1 × PBS and the flow-through is always discarded. The filter is then placed at a new vial, and 30 µl of 1 × PBS is added and the column is slightly vortexed. After this the filter is turned around, centrifuged at 1,500 rpm for 3 min, and the flow-through containing the biotinylated cells is used for further analysis.

4.1.2. Microarray-Based Analysis (see Note 2)

1. Prior to reaction with *E. coli* cells (see earlier), each ArrayTube is washed twice with 500 µl PBS–Tween wash buffer.
2. The microarray is blocked with 100 µl of freshly prepared PBS–Tween–FCS ***(10)*** for 15 min and washed with 500 µl PBS–Tween for 5 min. All the three steps are performed at 23°C and 550 rpm in the rotary shaker.

3. An aliquot of biotinylated or non-labelled *E. coli* cells [30 or 10 µl, respectively (see earlier)] is mixed with PBS–FCS–Tween to a final volume of 100 µl, added into the AT, and incubated at 23°C and 400 rpm for 15 min.
4. After removing the sample carefully the microarray is washed in PBS–Tween for 5 min.
5. If the non-biotinylated cells are used, 100 µl of anti-*E. coli* core LPS monoclonal antibody WN1222-5 is applied at 23°C and 400 rpm for 20 min. After that, the array is washed with PBS–Tween at 23°C and 400 rpm for 5 min.
6. 100 µl of a secondary biotinylated anti-mouse IgG is added and incubated at 23°C and 550 rpm for 10 min.
7. The microarray is again washed with PBS–Tween at 23°C and 550 rpm for 10 min.
8. For conjugation, 100 µl of streptavidin-poly-horseradish peroxidase (SA-poly-HRP) is added to the ArrayTube.
9. After 15- min conjugation at 30°C and 550 rpm, two washing steps using 500 µl PBS–Tween at 23°C and 550 rpm for 5 min are carried out.
10. The solution is completely removed and the microarray is stained at 25°C for 10–15 min by adding 100 µl of a precipitation substrate of streptavidin-poly-horseradish peroxidase, e.g., Seramun green, followed by the readout of the resulting picture using the atr01 or atr03 reader (CLONDIAG, Jena, Germany) according to manufacturers' instructions.
11. If the biotinylated cells were used, the incubation with primary and secondary antibody and subsequent washing steps were omitted with otherwise the same protocol.

4.2. Antibody Screening and Epitope Mapping

4.2.1. Antibody Purification and Labelling with Biotin

1. The protein concentration of the purified antibody to be labelled is determined by measuring the extinction at 280 nm. Afterwards, the concentration is adjusted to 1 mg/ml with 1 × PBS buffer.
2. To purify the antibody from interfering substances, 100 µl of the 1 mg/ml solution is mixed with 300 µl 1 × PBS buffer, added to a Microcon YM-30 column, and centrifuged at 14,000 rpm for 10 min. The flow-through is discarded, 400 µl 1 × PBS is added, and a second centrifugation step is performed at 14,000 rpm for 10 min. The filter is then placed at a new vial and the column is slightly vortexed. After this step, the filter is turned upside down and centrifuged at 1,500 rpm for 3 min. The flow-through is collected and adjusted to 100 µl with 1 × PBS.

3. 1.5 µl of NHS-LC-biotin solution is mixed with 100 µl of the purified antibody solution and stored at 21°C for 30 min. Ten microlitres 1 × PBS is added and the solution is divided into two 55 µl aliquots. To remove free biotin, these 55 µl aliquots are applied to two ZEBA spin columns and centrifuged at room temperature at 1,500 rpm for 1 min. The flow-through contains the biotinylated antibody and can be used in microarray experiments.

4.2.2. Microarray-Based Epitope Mapping

1. The ArrayTubes with spotted peptides are washed with 500 µl PBS–Triton for 2 min at 500 rpm and 37°C on a rotary shaker.
2. The biotinylated antibody is applied to the peptide microarray in a final concentration between 10 and 100 ng/ml in a streptavidin-buffer at 37°C and 800 rpm for 15 min.
3. The microarray is washed at 37°C, 800 rpm and for 1 min subsequently once with 500 µl 1 × PBS + 0.1% Triton X100 and twice with 1 × PBS.
4. After removing the wash buffer, the TMB staining is performed by adding 100 µl of True Blue peroxidase substrate, incubating at 25°C (no shaking!) for 10 min and subsequent image recording and analysis with the atr03 reader device (CLONDIAG, Jena, Germany).

4.2.3. Microarray-Based Antibody Screening (see Note 2)

1. The ArrayTubes with spotted antibodies to be screened are washed with 500 µl PBS–FCS–Tween, at 37°C and 400 rpm for 5 min using a rotary shaker.
2. A blocking step follows using 500 µl PBS–Tween–FCS ***(10)*** at 37°C and 500 rpm for 5 min.
3. After removal of the blocking solution a characterised human serum in a dilution range between 1:10^6 and 1:1,000 in 1 × PBS is applied at 500 rpm and 37°C for 30 min followed by a 5- min washing step with 500 µl PBS–FCS–Tween at 400 rpm and 37°C.
4. The biotinylated detection antibody is applied in a dilution between 1:500 to 1:10,000 in 100 µl PBS–FCS–Tween at 300 rpm and an incubation at 37°C for 10 min followed again by a 5- min washing step with 500 µl PBS–FCS–Tween at 400 rpm and 37°C.
5. For conjugation streptavidin poly-HRP (0.2 ng/ml) is used at 400 rpm and 37°C for 10 min followed by a 5- min washing step with 500 µl PBS–FCS–Tween at 400 rpm and 37°C.
6. After removal of the washing solution, 100 µl of the precipitating substrate seramun green is added and the microarrays are incubated at 25°C without shaking for 10 min. After-

wards, the resulting picture is taken and analysed using the atr03 reader in combination with the Iconoclust Software package and an assay-specific script.

4.3. Detection of Staphylococcal Enterotoxin B

4.3.1. Bacterial Culture (see Note 1)

1. *S. aureus* is grown in liquid media based on a publication by Noda, Hirayama, Kato, and Matsuda *(19)*.
2. One Microbank bead (Viva Diagnostika, Cologne, Germany) of a stored culture, or one inoculation loop of a fresh one, is inoculated into 1.5 ml of medium and incubated for 12 h on a shaker using test tubes with cotton plugs in order to facilitate oxygen influx. Then, cultures are transferred into Eppendorf tubes and centrifuged (7,000 rpm, 10 min) to obtain cell-free supernatants.
3. Prior to use with the antibody array, the supernatants are diluted either 1:10 or 1:50 in 1 × PBS. Applying this procedure on *seB*-positive reference strain COL *(20)*, it is possible to yield a working concentration of approximately 0.01–0.05 µg/ml enterotoxin B.
4. To detect enterotoxin B from spiked minced meat, or from minced meat inoculated with *seB*-positive strains such as COL, an equal amount of PBS–Tween (see later) is added. After vigorous vortexing, the suspension is centrifuged (15,000 rpm, 10 min).
5. The supernatant is filtered using 0.2 µm syringe filters. The resulting clear solution is used for further experiments with the antibody array. When incubating reference strain COL with minced pork meat for 60h at 37°C, approximately 0.001 µg/ml enterotoxin B is yielded after elution, centrifuging, and filtering.

4.3.2. Array Procedure (see Note 2)

All incubation or washing steps are carried out at 25°C using a thermo shaker set at 300 rpm.

1. The ArrayTube is washed using 500 µl PBS–Tween for 5 min.
2. Unspecific binding capacities are blocked by incubation with 100 µl PBS–Tween–BSA for 5 min.
3. The culture supernatant or toxin preparation (see earlier) is added and incubated for 20 min.
4. The microarray is washed with PBS–Tween–BSA for 5 min.
5. After removal of the washing buffer, 100 µl biotinylated antibody BBC202 (1:2,000) is added and the ArrayTube is incubated for 20 min.
6. The microarray is washed again with PBS–Tween–BSA for 5 min.

7. 100 ml diluted Streptavidin-HRP (1:10,000, see earlier) is added and the ArrayTube is incubated for 20 min.
8. The microarray is washed twice (2 × 5 min) with PBS–Tween.
9. Hundred microlitres of a precipitating substrate for staining is added. The ArrayTube is incubated for another 10 min and images are recorded using a atr01 reader.

4.4 .Detection of Biological Warfare Agents

4.4.1. Antigen Preparation (see Note 1)

Viruses:

1. Alpha- and flaviviruses are either grown in Vero or BHK 21 cells in the biosafety level 2 and 3 facilities. Virus titres are determined by the 50% tissue culture infective dose ($TCID_{50}$/ml) method *(27, 28)*.
2. All pathogeneic viruses are inactivated prior to use. Inactivation of viruses is performed by incubation with 0.1% β-propiolactone for 1 h at 4°C and 4 h at 37°C.

Bacteria:

1. Bacterial strains are cultured according to standard cultivation procedures.
2. Inactivation of bacteria is achieved either by formaldehyde, heat incubation, or by a combination of both.

Toxins:

1. Toxins are to be handled as required by national legislation (Ordinance on safety and health protection related to work involving biological agents including toxic substances, BioStoffV).
2. Crude extracts of Ricin are prepared from castor beans (*Ricinus communis*) by aqueous extraction and ammonium sulphate precipitation *(29)*, using a modified protocol (Binder, Central Institute of the German Armed Forces Medical Corps, Munich, 2003, personal communication).

4.4.2. Biotinylation of Detection Antibodies

1. All mono- and polyclonal antibodies applied for the specific detection of BWAs are coupled to biotin-NHS ester.
2. Incubation is performed for 2 h at room temperature according to the manufacturer's direction.
3. Unincorporated biotin is removed by gel filtration on PD-10 columns.
4. Positive detection reactions are reported by streptavidin–HRP conjugates.
5. To minimise unspecific cross reactions, each detection antibody is titrated prior to regular use.

4.4.3. Array Analysis (see Note 2)

1. All incubation steps of the analysis are carried out on a horizontal tube shaker at 350 rpm and 25°C.
2. Before starting a protein array analysis all ArrayTubes are conditioned by washing them twice with 500 µl PBS–Tween for 2 min.
3. To block unspecific binding sites, the ATs are incubated in PBS–FCS–Tween + 1% fat-free milk powder or in PBS–Tween containing 1% FCS for 15 min.
4. This incubation is followed by three washing steps with 500 µl 1 × PBS–Tween for 2 min each.
5. Antigen binding is allowed to proceed in PBS–FCS–Tween for 30 min.
6. The ATs are washed three times as described earlier.
7. The incubation of ATs with specific biotinylated secondary antibodies is performed in PBS–FCS–Tween for 30 min.
8. After three washing steps as described earlier, specific binding of the secondary antibody is detected by Streptavidin-poly-horseradish peroxidase and TMB staining. SA-poly-HRP is used in a 1:10,000 dilution.
9. Online read-out of washed ATs is performed in an ArrayTube reader (atr01) for 6 min at 25°C, recording one image every 10s.
10. Data analysis is done with the manufacturers' specifications and the IconoClust™ software.

5. Notes

1. The microorganisms described herein are potentially dangerous pathogens, which need to be cultured at least under biosafety level 2 conditions. All experiments are to be performed by experienced staff in an appropriate facility.
2. Human sera and other specimens are potentially infectious because of possible contamination with HIV, HBV, etc. Thus, human specimens need to be handled using protective gloves.
3. Recombinant enterotoxins and ricin may cause pulmonary edema, as well as other severe conditions upon inhalation, ingestion, or eye/skin contact. Thus, toxins are to be handled using protective gloves, goggles, and masks. Handling of these substances might be regulated by national legislation (in Germany, "Ordinance on Safety and Health

Protection Related to Work Involving Biological Agents Including Toxic Substances, BioStoffV").

4. The ArrayTubes (ATs) are stored in a light-protective foil pouch sealed under inert gas (argon). Within these packages, the protein microarrays can be stored at 4°C or room temperature. When a pouch was opened, humidity, direct sunlight, and dust should be avoided, and the ATs should be used within the next 24h.
5. Never let the microarrays run dry during processing.
6. Before adding a new solution into ATs, always carefully remove precedent solution by using a fine pipette without touching the array at the bottom of the AT.
7. Follow standard lab safety regulations when using the ATs with any hazardous or potentially infectious material that may be required for your individual experiments.
8. Do not expose the ATs to direct sunlight.
9. Avoid scratching or touching the array surface (e.g. during pipetting steps with pipette tip).
10. Do not centrifuge the ArrayTubes.
11. Do not heat ArrayTubes over 60°C for a longer time period.
12. Avoid the deposition of dust particles and/or filaments on the outer side of the AT array. In case you detect any dust or filaments, carefully remove it with a dust-free cloth moisturised with 70% ethanol.
13. Avoid formation of air bubbles during all assay steps and remove them, if necessary, by mixing with a pipette or by short agitation.
14. Pre-warm the portion of TMB or seramun green solution needed for the actual experiments to 25°C prior to use. Then, shortly (ca. 15s) centrifuge the solution at 5,000 rpm before finally adding it to the processed ATs for staining.
15. Prepare all blocking reagents such as FCS or milk powder solutions always freshly prior to use.
16. Always use minimum volumes of 90–100 µl per step within the different protocols.
17. Some batches of fetal calf serum may degrade biotin spots. Thus, it is recommended to test new brands or batches of FCS prior to use in crucial or large-scale experiments.

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